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The role of estrogen receptor alpha in mediating chemoresistance in breast cancer cells

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Abstract

Introduction: Previous studies suggested that estrogen receptor alpha (ERα) plays an important role in the chemoresistance of breast cancers. However, large random trials failed to demonstrate any benefit of the concurrent estrogen antagonist tamoxifen on the chemotherapy efficacy. Thus, in the present study, the importance of the role of ERα in the chemoresistance of breast cancer cells was investigated.

Methods: The ERα-transfected Bcap37 cells and natural ERα-positive T47D breast cancer cells were treated using chemotherapeutic agents with or without 17-beta estradiol (E2) pretreatment. Their viabilities were assessed using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assays. The dead cell rates were determined using propidium iodide dye exclusion tests, and the expression levels of Bcl-2 and Bax were detected through Western blot analysis. The effects of E2 on the growth of breast cancer cells were also determined via cell growth curve and cell cycle analysis.

Results: ERα activation by E2 increased the sensitivity of natural ERα-positive T47D breast cancer cells to chemotherapeutic agents. However, the increase in ERα expression in ERα-negative Bcap37 breast cancer cells also significantly increased their resistance. These phenomena cannot be explained by asserting that ERα mediated the chemoresistance of breast cancer cells by regulating the expression of Bcl-2 and Bax. Our findings show that ERα activation upregulated the expression of Bcl-2 in natural ERα-positive T47D breast cancer cells, whereas ERα activation by E2 downregulated and upregulated the Bcl-2 and Bax expression levels, respectively, in ERα-transfected Bcap37 cells. This phenomenon was due to the influence of ERα on the growth of breast cancer cells. Specifically, ERα activation enhanced the growth of natural ERα-positive breast cancer cells and thus increased their sensitivity to chemotherapeutic agents. However, ERα activation also inhibited the growth of ERα-transfected Bcap37 cells and increased the resistance of cancer cells to chemotherapeutic agents. Chemoresistance of ERα-transfected Bcap37 cells was only due to the specific growth inhibition by E2, which is not applicable to common ERα-positive breast cancer cells.

Conclusions: Although ERα was associated with chemoresistance of breast cancers, ERα itself did not mediate this resistance process.

Keywords: Estrogen receptor alpha, Chemoresistance, Breast cancer, Bcl-2, Cell growth

Background

Estrogen Receptors alpha (ERα) are expressed in approximately 65% of breast cancer cases. Binding of estrogen (such as estradiol) to ERα induces tumor growth in most ERα-positive breast cancer cell lines [1]. Active Estrogen Receptors alpha can also inhibit apoptosis of breast cancer cells by upregulating Bcl-2 expression [2]. Fulvestrant is a novel ERα antagonist with no agonist effects. It binds ERα, prevents dimerisation, and leads to the rapid degradation of the fulvestrant–ERα complex, downregulating cellular ERα levels [3].

Our and other studies have suggested that ERα-positive breast cancer is more resistant to chemotherapy than ERα-negative cancer [4-9]. In vitro studies have also shown that ERα plays an important role in determining the sensitivity of breast cancer cells to chemotherapeutic agents [2,10-14]. Considering the observed consistency between previous clinical and in vitro findings, it seems reasonable that ERα mediates the chemoresistane of breast cancer cells. Does ERα really mediate the chemoresistance of breast cancer cells? We think this problem needs further investigation, because other clinical studies have

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failed to show any benefit of concurrent tamoxifen on the chemotherapy efficacy [15-17]. The proliferation index (Ki-67) correlates well with chemotherapy response; in addition, slowly growing breast cancer is resistant to chemotherapy [18-20]. However, ERα-positive breast cancer grows more slowly than an ERα-negative one [21]. This gives rise to the question "Is it tumor growth rate (and not ERα expression), which determines the chemosensitivity of breast cancer?" To understand whether or not ERα actually mediates drug resistance to chemotherapy in breast cancer, an in vitro study was performed by us to determine the relationship between ERα and drug resistance to chemotherapeutic agents in breast cancer cells.

Results

Activation of ERα by 17-βestradiol (E2) increased the sensibility of ERα-positive T47D cells to chemotherapeutic agents and fulvestant reversed the effect of E2

3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assays were performed to determine the viability of T47D cells treated with four different chemotherapeutic agents (i.e., paclitaxel, epirubicin, fluorouracil, and vinorelbine) with or without the pretreatment of E2. Three concentrations were tested for each chemotherapeutic agent. As shown in Figure 1A and 1B, the pretreatment of 100 nM E2 for 16 hours or 12 days significantly decreased cell survival after exposure to

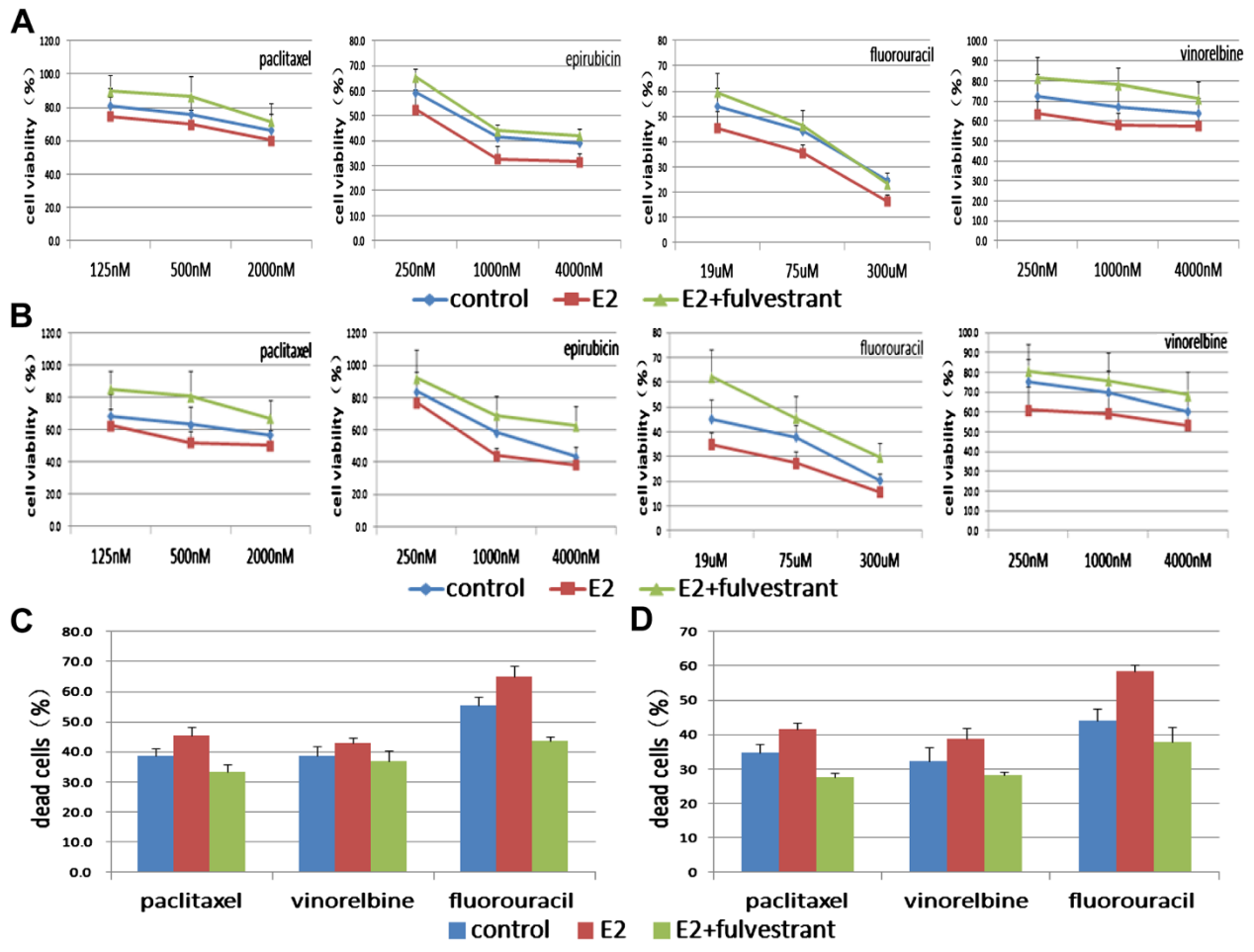

Figure 1 Activation of ERα increased the sensibility of T47D cells to chemotherapeutic agents. (**A**, **B**) The viability of T47D cells after being exposed to four chemotherapeutic agents was determined by MTT assays. (**A**) Cells were pretreated with or without E2 for 16 hours before being exposed to chemotherapeutic agents. (**B**) Cells were pretreated with or without E2 for 12 days. Fulvestrant was added to the medium 12 hours before E2 treatment. The chemotherapeutic agents used in the MTT assays were paclitaxel, epirubicin, fluorouracil, and vinorelbine. Three concentrations were tested for each chemotherapeutic agent. Data are means ± standard deviation (SD) (n = 3). (**C**, **D**) Cell death induced by chemotherapeutic agents was determined by PI dye exclusion assays. (**C**) Cells were pretreated with or without E2 for 16 hours before exposed to chemotherapeutic agents. (**D**) Cells were pretreated with or without E2 for 12 days. Fulvestrant was added to the medium 12 hours before E2 treatment. The chemotherapeutic agents used in the PI dye exclusion assays were paclitaxel, fluorouracil, and vinorelbine. One concentration was tested for each chemotherapeutic agent. Bars correspond to mean ± SD.

chemotherapeutic agents ($p < 0.05$). To determine whether or not the E2-induced chemosensitivity was specifically due to an ERα-mediated mechanism, fulvestrant (an ERα antagonist) was used 12 hours before E2. We found that pretreatment with 2 uM fulvestrant completely reversed E2-induced sensitivity to chemotherapeutic agents ($p < 0.05$).

To confirm the effect of ERα on the chemosensitivity of T47D cells, the occurrence of chemotherapeutic agent-induced cell death was assessed using propidium iodide (PI) dye exclusion tests. The chemotherapeutic agents used in the PI dye exclusion tests were paclitaxel, fluorouracil, and vinorelbine. Epirubicin spontaneously emits red fluorescent light, and the wavelength of fluorescent light is similar to that of PI, which interferes with the detection of dead cells induced by epirubicin. Thus, epirubicin was not used in the PI dye exclusion tests performed for the current work. One concentration was tested for each chemotherapeutic agent. As shown in Figure 1C and 1D, the pretreatment of E2 for 16 hours or 12 days significantly increased the cell death induced by chemotherapeutic agents, such as paclitaxel, fluorouracil, and vinorelbine ($p < 0.05$). Moreover, fulvestrant reversed the enhancing effect of E2 on the chemotherapeutic agents-induced cell death ($p < 0.05$).

Treatment of ERα-positive T47D cells with E2 up-regulated the expression of the bcl-2 protein

The experimental results in this work showed that ERα mediated chemosensitivity in T47D cells. However, some reports have shown that ERα mediated chemoresitance in breast cancer cells through the regulation of Bcl-2 family [2,10,11,13,14]. ERα-positive breast cancer cells usually express Bcl-2, whereas ERα-negative ones express little or no Bcl-2 [22,23]. We investigated the expressions of Bcl-2 and Bax in T47D cells after incubation with E2 and/or fulvestrant for 12 days in order to determine whether Bcl-2 family contributed to ERα-mediated chemosensitivity. As shown in Figure 2, the treatment of T47D cells with E2 for 12 days resulted in a marked increase in Bcl-2 expression, and fulvestrant reversed the upregulation of Bcl-2. Bax protein was undetectable in T47D cells grown in an E2-free medium or in a medium supplemented with 100 nM E2 for 12 days. Considering the antiapoptotic function of Bcl-2, these results suggested that ERα-mediated chemosensitivity in T47D cells was not due to Bcl-2 alteration induced by E2.

Treatment with E2 enhanced the growth of T47D cells, whereas fulvestrant inhibited its growth

The cell cycle plays a critical role in chemosensitivity, particularly for cycle-specific chemotherapeutic agents. High levels of cell proliferation normally lead to increased sensitivity to chemotherapeutic agents. Since apoptosis-related protein Bcl-2 and Bax do not contribute to ERα-mediated chemosensitivity in T47D cells, we investigated the role of cell cycle alteration in this process. The results presented in Figure 3A and 3B show that E2 treatment for 16 hours decreased the percentage of T47D cells in G1 phase, as compared with the cells grown in the absence of E2, with a concomitant increase in S and G2/M phase population. E2 treatment for 12 days led to greater accumulation of cells in the S and G2/M phases. E2 induced an increase in the proliferative potential of T47D cells, which was demonstrated by the growth curve. In addition, E2 promoted T47D cell growth significantly compared with the control cell group. Fulvestrant completely inhibited E2-induced cell proliferation. We postulated that it was cell cycle alteration, and not the Bcl-2/Bax apoptotic pathway, which was involved in ERα-mediated chemosensitivity of T47D cells.

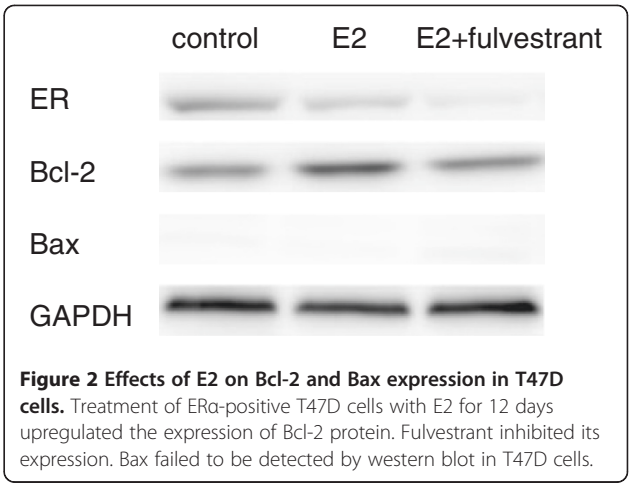

Figure 2 Effects of E2 on Bcl-2 and Bax expression in T47D cells. Treatment of ERα-positive T47D cells with E2 for 12 days upregulated the expression of Bcl-2 protein. Fulvestrant inhibited its expression. Bax failed to be detected by western blot in T47D cells.

ERα transfected Bcap37 cells (BC-ER cells) exhibited much higher resistance to chemotherapeutic agents than cells transfected with empty vector (BC-V cells) in the presence of E2.

The stable transformants of the Bcap37 cells were established after transfection with either ERα expression vector (BC-ER cells) or empty vector (BC-V cells). The difference in chemosensitivity between BC-ER cells and BC-V cells was determined by MTT assays and PI dye exclusion tests. This process was completed after the cells were exposed to chemotherapeutic agents for 72 hours with or without preincubation of 10 nM E2 for either 16 hours or 12 days.

In the absence of E2, BC-ER and BC-V cells exhibited similar cell viability. However, in the presence of E2, cell viability after treatment using chemotherapeutic agents was much higher in BC-ER cells than in BC-V cells ($P < 0.05$; see Figure 4A and 4B). Pretreatment with E2 for 16 hours or 12 days increased the cell viability of BC-ER cells after exposure to chemotherapeutic agents.

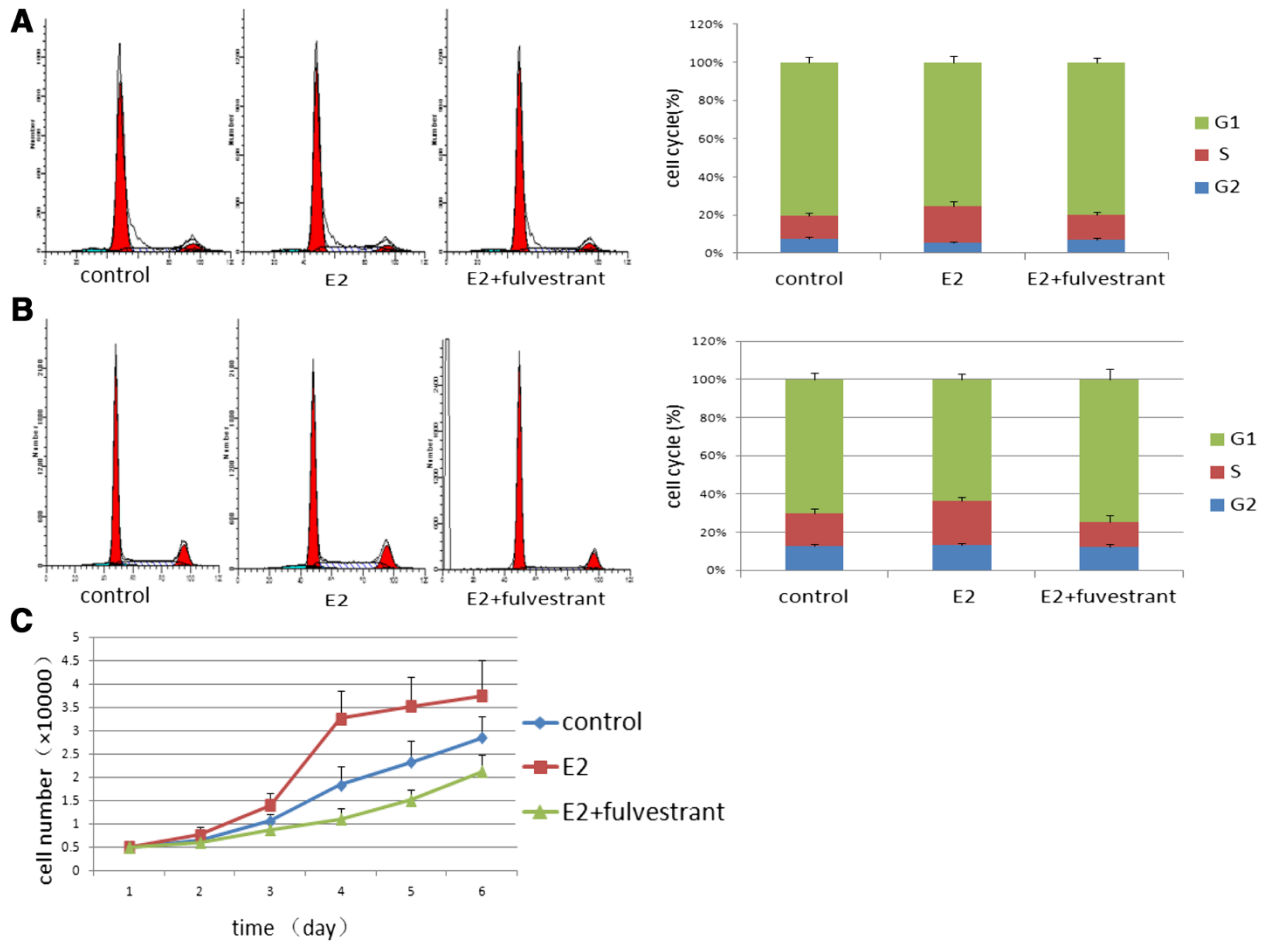

Figure 3 Treatment with E2 enhanced the growth of T47D cells and fulvestrant inhibited its growth. (**A**, **B**) Influence of E2 or fulvestrant on the cell cycle status of T47D cells. (**A**) Cells were treated with E2 for 16 hours before being analyzed by flow cytometry. (**B**) Cells were treated with E2 for 12 days. Fulvestrant was added to the medium 12 hours before E2 treatment. (**C**) The growth curve of E2 or fulvestrant treated T47D cells and control cells were plotted for 6 days of cell culture.

Results of PI dye exclusion tests showed that in the absence of E2, BC-ER and BC-V cells had similar levels of cell death after treatment with chemotherapeutic agents. However, in the presence of E2, the percentage values of PI-stained dead cells were significantly lower in BC-ER than in BC-V cells. In contrast to the action of E2 on the death of T47D cells induced by chemotherapeutic agents, pretreatment of BC-ER cells with E2 for either 16 hours or 12 days decreased cell death significantly (Figure 4C and 4D).

BC-ER cells showed lower Bcl-2 expression and higher Bax expression than BC-V cells in the presence of E2

We investigated the mechanism of the resistance of BC-ER cells to chemotherapeutic agents. Western blot was performed to determine the protein expression of Bcl-2 and Bax in BC-ER and BC-V cells in the presence or absence of E2. In contrast to the effect of E2 on Bcl-2 expression in T47D cells, treatment with E2 for 12 days decreased the expression level of Bcl-2 significantly. BC-ER cells had lower Bcl-2 expression than BC-V cells when treated with E2 for 12 days. Low Bax expression levels were detected in both BC-ER and BC-V cells; however, treatment with E2 induced an increase of Bax expression in BC-ER cells (Figure 5).

BC-ER cells showed a lower Bcl-2/Bax ratio than BC-V cells in the presence of E2, which did not contribute much to greater resistance of BC-ER cells than BC-V cells.

BC-ER cells grew more slowly than BC-V cells in the presence of E2

Since the Bcl-2/Bax apoptotic pathway did not contribute to the chemoresistance of BC-ER cells, we investigated the role of cell growth rate in the development of chemoresistance in BC-ER cells. In contrast to the

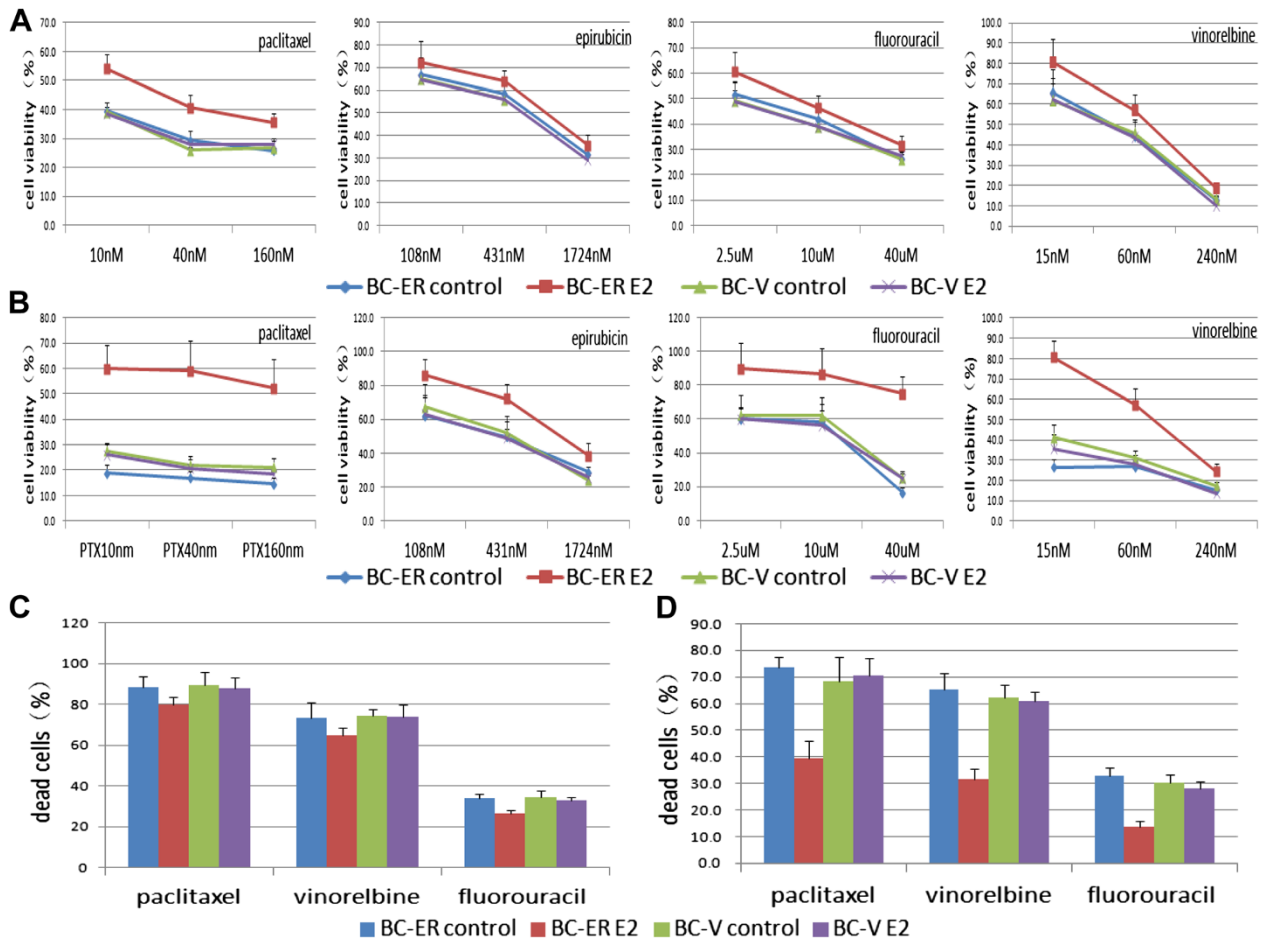

Figure 4 BC-ER cells exhibited much higher resistance than BC-V cells in the presence of E2. (**A**, **B**) The viability of BC-ER and BC-V cells after being exposed to four chemotherapeutic agents was determined by MTT assays. (**A**) Cells were pretreated with or without E2 for 16 hours before being exposed to chemotherapeutic agents. (**B**) Cells were pretreated with or without E2 for 12 days. The chemotherapeutic agents used in the MTT assays were paclitaxel, epirubicin, fluorouracil, and vinorelbine. Three concentrations were tested for each chemotherapeutic agent. (**C**, **D**) Cell death induced by chemotherapeutic agents was determined by PI dye exclusion assays. (**C**) Cells were pretreated with or without E2 for 16 hours before being exposed to chemotherapeutic agents. (**D**) Cells were pretreated with or without E2 for 12 days. The chemotherapeutic agents used in the PI dye exclusion assays were paclitaxel, fluorouracil, and vinorelbine. One concentration was tested for each anticancer drug.

effect of E2 on T47D cells, E2 treatment for 16 hours increased the percentage of BC-ER cells in the G1 phase and decreased the percentage of cells in the S and G2/M phases. E2 treatment for 12 days led to a marked accumulation of cells in the G1 phase. E2 treatment had no obvious influence on cell cycle distribution of BC-V cells. The percentages of BC-ER cells in the S and G2/M phases were significantly lower than those of BC-V cells. E2 inhibited the proliferation of BC-ER cells as demonstrated by the growth curve. However, the growth of BC-V cells was not influenced by E2 treatment (Figure 6). In the presence of E2, BC-ER cells had lower growth potential than BC-V cells, which may have induced the resistance of BC-ER cells to chemotherapeutic agents.

Discussion

Several studies have reported the relationship between ERα and resistance to chemotherapeutic agents in breast cancer cells [2,10-14]. Most papers have reported the activation of ERα by E2 upregulated expression of Bcl-2, which leads to resistance to chemotherapeutic agents in breast cancer cells. MTT assays have always been used to judge resistance to paclitaxel in these studies. The limitation of some studies is that these co-culture breast cancer cells with paclitaxel for only 24 hours before MTT assays, while the initial effect of paclitaxel is obtained slowly [2]. In our opinion, it is more appropriate to treat cells with paclitaxel for 72 hours. Moreover, in some studies, inappropriate control groups have been set up, leading to deviations in the results [2,10-12,14].

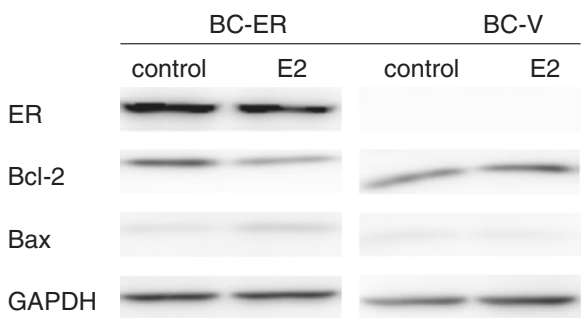

Figure 5 Bcl-2 and Bax protein expression in BC-ER and BC-V cells. BC-ER cells showed lower Bcl-2 expression and higher Bax expression than BC-V cells in the presence of E2 (western blot). Treatment of BC-ER cells with E2 for 12 days downregulated Bcl-2 and upregulated the Bax expression.

Some researchers have observed that drug resistance increases after ERα-negative breast cancer cells are transformed into ERα-positive breast cancer cells, indicating that ERα mediates chemoresistance in breast cancer [11,13,14]. However, such works did not consider significant differences in biological behavior between natural ERα-positive breast cancer cells, and ER-positive breast cancer cells established by plasmid transfection. Furthermore, the relationship between ERα and drug resistance has been analyzed only from the mechanism of apoptosis regulation, without considering the influence of the proliferation rate of tumor cells on chemoresistance. We think that the conclusions from these studies are not applicable for normal ERα-positive breast cancer cells.

In the present work, we used MTT methods and PI dye exclusion tests to evaluate the effects of ERα on the sensitivity of breast cancer cells to chemotherapeutic agents [24]. MTT results showed that the sensitivities to all the four kinds of chemotherapeutic agents improved

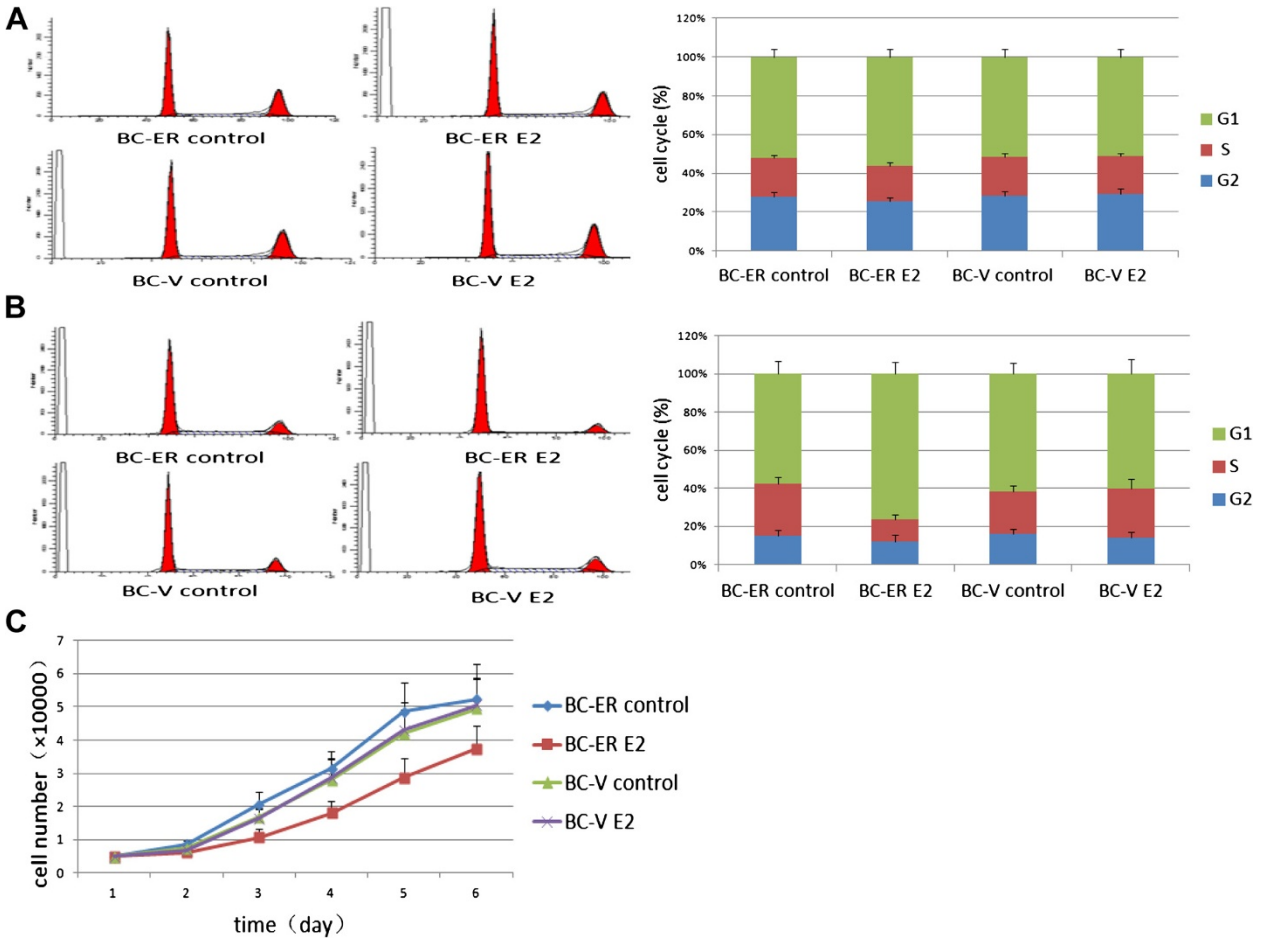

Figure 6 BC-ER cells grew more slowly than BC-V cells in the presence of E2. (**A**, **B**) Cell cycle status of the BC-ER and BC-V cells. (**A**) Cells were treated with E2 for 16 hours before being analyzed by flow cytometry. (**B**) Cells were treated with E2 for 12 days. (**C**) The growth curve of the BC-ER and BC-V cells was plotted for 6 days of cell culture.

in natural ERα-positive T47D cells under the action of E2. The sensitizing effect of E2 was more significant when the cells were pretreated with E2 for 12 days, while fulvestrant reversed the sensitizing effect of E2. It is worth noting that the computational formula of cell survival rate in our MTT assays was as follows: cell survival rate = OD value of chemotherapeutic agent group / OD value of the corresponding control group × 100% (i.e., cell survival rate of simple chemotherapeutic agent group = OD value of the chemotherapeutic agent group / OD value of the control group × 100%, cell survival rate of E2 + chemotherapeutic agent group = OD value of E2 + chemotherapeutic agent group / OD value of E2 group × 100% (rather than OD value of the control group). In this way, the effects of E2 and fulvestrant on the growth of breast cancer cells were not involved in the resistance of chemotherapeutic agents, making the results more accurate and reliable.

The results of PI dye exclusion tests also demonstrated the chemosensitizing effect of E2 in ERα-positive breast cancer cells. The number of dead cells induced by chemotherapeutic agents increased in T47D breast cancer cells after pretreatment with E2. However, the number of dead cells was significantly decreased in the presence of both fulvestrant and E2, indicating resistance to chemotherapeutic agents.

Thus, it was clear that ERα was not able to mediate resistance to chemotherapeutic agents in T47D breast cancer cells; rather, it increased sensitivity to chemotherapeutic agents in these cells. We investigated the possible role of the Bcl-2/Bax apoptosis pathway in the chemosensitizing effect of ERα. Bcl-2/Bax plays an important role in the regulation of apoptosis [25,26]. The expression changes of Bcl-2 and Bax under the action of E2 and fulvestrant were detected by western blot. The results showed that Bcl-2 expression in T47D cells increased after being treated with E2 for 12 days and that fulvestrant inhibited Bcl-2 expression, which was consistent with the results reported by other studies. However, the expression changes of Bcl-2 failed to explain the chemo-sensitizing effects of E2 on T47D cells. The expression of Bax protein was not detected in T47D cells by western blot. Then, which mechanism was involved in the sensitivity changes of chemotherapy in T47D cells? Cell proliferation rate is an important factor affecting chemosensitivity of a malignant tumor, that is, the higher growth fraction of tumor cells (the ratio of the cells in G2 + S period), the higher the sensitivity to chemotherapy [27,28]. The ratio of the cells in the G2 + S period increased after being treated with E2 for 16 hours or 12 days. E2-inducing increase in the proliferative potential of T47D cells was also demonstrated by growth curve, while fulvestrant completely reversed such growth-promoting effect. The growth-promoting effect of E2 may have led to the sensitivity of ERα-positive T47D cells to chemotherapeutic agents.

Thus, we know that the activation of ERα failed to enhance resistance of natural ERα-positive T47D breast cancer cells to chemotherapeutic agents. During the following experiments, plasmid-expressing ERα was stably transfected into ERα-negative human breast cancer cells (BCap37) to establish ERα-expressing BCap37 cells (BC-ER). Both BC-ER cells and BCap37 BC-V cells were used to study the relationship between ERα and resistance to chemotherapeutic agents. In the absence of E2, sensitivity to chemotherapeutic agents was similar in both BC-ER and BC-V cells. In the presence of E2, significant resistance to chemotherapeutic agents existed in BC-ER cells. E2 pretreatment increased the resistance of BC-ER cells to chemotherapeutic agents.

What caused resistance to chemotherapeutic agents in ERα-positive BC-ER cells? We investigated the expression of apoptosis-regulating proteins Bcl-2 and Bax in BC-ER and BC-V cells. In contrast to natural ERα-positive T47D cells, the expression of Bcl-2 was reduced in BC-ER cells after being treated with E2 for 12 days, while the expression of Bax was upregulated. In addition, there was no significant change in BC-V cells. Such abnormal expression of apoptosis-regulating proteins under E2 action has not yet been reported in literature. Resistance to chemotherapeutic agents is difficult to explain in BC-ER cells with apoptosis-regulating proteins, such as Bcl-2 and Bax. Subsequently, the cause of drug resistance in BC-ER cells was analyzed by considering cell proliferation. In contrast to T47D cells, BC-ER cells grew slower after being treated with E2, and cell proportion in the G2 + S period was reduced. This result is consistent with previous studies showing that E2 inhibits the growth of ERα-positive breast cancer cells transformed from ERα-negative cells [29-31]. We supposed that drug resistance of BC-ER cells was due to its low growth velocity in the presence of E2. However, the apoptosis-regulating proteins Bcl-2 and Bax, which are considered as important proteins mediating drug resistance in ERα-positive breast cancer cells, may not play a role in the formation of drug resistance of BC-ER cells.

The results obtained above showed that ERα activation increased the sensitivity of natural ERα-positive T47D breast cancer cells to different chemotherapeutic agents, and that the inhibition of ERα activation by fulvestrant resulted in chemoresistance. Meanwhile, ERα activation decreased the chemosensitivity of ERα-stably transfected BC-ER cells. Compared with ERα-negative BC-V cells, ERα-positive BC-ER cells presented higher resistance to multiple chemotherapeutic agents. We could not explain these phenomena by stating that ERα mediated the drug resistance of breast cancer cells to chemotherapy through the regulation of the expression of Bcl-2 and Bax. This is

because ERα activation upregulated the expression of Bcl-2 in natural ERα-positive breast cancer cells, however, ERα activation downregulated Bcl-2 expression and upregulated Bax expression in ERα-positive cancer cells transformed from ERα-negative breast cancer cells. We explained this phenomenon through the influence of ERα on the growth of breast cancer cells, that is, ERα activation enhanced the growth of natural ERα-positive breast cancer cells, and eventually increased sensitivity to chemotherapeutic agents. However, for Bcap37 cells transformed from ERα-negative breast cancer cells, ERα activation inhibited the growth of cancer cells, and increased the resistance of cancer cells to chemotherapeutic agents.

Conclusions

ERα activation was unable to induce the drug resistance of natural ERα positive T47D breast cancer cells. Although it increased the drug resistance of Bcap37 cells transformed from ERα-negative breast cancer cells, this was, however, attributable only to the inhibitory effect of E2 on the growth of these ERα-transfected Bcap37 cells. The observation was not applicable to common ERα-positive breast cancer cells. Taking together our in vitro and previous clinical findings, we indicated that, although ERα was associated with chemoresistance of breast cancers, ERα itself did not mediate this resistance process. This finding might explain why the co-application of the estrogen antagonist tamoxifen and the chemotherapeutic agents did not have good therapeutic effects in breast cancer therapy. Thus, we believe that reversing chemoresistance correlated with ERα using endocrine therapy drugs is inappropriate, although related studies have been performed by others [14].

Methods

Cell culture

T47D cells were obtained from ATCC, and Bcap37 cells were obtained from Cancer Institute, Zhejiang University. Bcap-37 is a ERα negative breast cancer cell line that first established in China. T47D, and Bcap37, and Bcap37, which were transfected with empty pcDNA3.1 expression vector (BC-V) or the pcDNA3.1- ERα expression vector (BC-ER), were cultured in RPMI 1640 supplemented with 10% newborn calf serum and 100 U/ml penicillin-streptomycin under 5% CO_2 atmosphere with humidity at 37°C. For estrogen induction assays, the cells were precultured in phenol red-free RPMI 1640 containing dextran-charcoal stripped 10% FBS (Hyclon) for 48 hours and then incubated with 17-βestradiol (Sigma) or ICI182780 (Sigma).

Cells were divided into 2 groups according to the preincubation time of 17-βestradiol (E2). In the short-term preincubation group, the cells were preincubated in phenol red-free RPMI 1640 medium containing dextran-charcoal stripped 10% FBS with or without E2 for 16 hours, before they were exposed to chemotherapeutic agents. In the long-term preincubation group, the cells were preincubated in RPMI 1640 medium with or without E2 for 12 days. For T47D cells, fulvestrant was added to RPMI 1640 medium 12 hours before E2 treatment. E2 was used at a concentration of 100 nM in T47D cells and 10 nM in Bcap37 cells. Fulvestrant was used at a concentration of 2 uM in T47D cells and 500 nM in Bcap37 cells.

Transfection

Cell transfection was carried out using Lipofectamine 2000, according to the instructions of the manufacturer. Briefly, ERα-negative BCap37 cells were placed in a six-well plate at a density of 1×10^6 cells/well and incubated overnight in RPMI 1640 supplemented with 10% FBS. PcDNA3.1-ERα or pcDNA3.1 plasmid DNA 4ug) was diluted in serum-free RPMI 1640 medium (250 ul) and then mixed with the transfection solution for 15 min. Then, 24 hours after transfection, the transfectants were selected by incubation in a medium containing G418 (500 ug/ml), until positive clones were discovered after 2–3 weeks. Positive clones were maintained in a medium supplemented with 200 ug/ml G418.

Measurement of cell viability by MTT assays

Cells were seeded at a density of 8000 cells/well for T47D cells or 5000 cells/well for Bcap37 cells in 96-well microplates. The cells were then treated with four chemotherapeutic agents, including paclitaxel, epirubicin, fluorouracil and vinorelbine, after preincubation with E2 or fulvestrant. At the end of the culture, 20 ul 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 5 mg/ml) were added to each well, and plates were placed at 37°C for 4 hours. Then, 150 ul of dimethylsulfoxide was added to each well to lyse the cells. Absorbance was measured at 570 nm using a microplate reader.

Measurement of dead cell rate through the PI dye exclusion tests

The dead cell rate was determined by PI dye exclusion tests. Propidium iodide (PI) was excluded from live cells, whereas dead cells were stained positive for PI. Cells were harvested after being treated with chemotherapeutic agents for 72 hours; these were suspended in PBS and then mixed with PI. The cells were then analyzed by flow cytometry. Results were expressed as percentages of PI fluorescent cells, which represented the percentages of dead cells.

Cell cycle analysis

The redistribution of cells in the cell cycle was analyzed by flow cytometry. After 12 days of cultivation, T47D

and Bcap37 cells were harvested by trypsinization, washed with PBS, and then fixed in 70% ethanol at 4°C for 24 hours. Cells were suspended in 1 ml of 0.1% Triton X-100 solution, incubated in 500 μl of propidium iodide solution (50 ug/ml) containing 250 ug of DNase-free RNase A, and analyzed for DNA content using a flow cytometer (Beckman Coulter EPICS XL, USA).

Growth curve

Breast cancer cells (5×10^3 cells per well) were plated in 24-well tissue culture plates. Cells were collected by trypsinization every day until day 6. The total cell number was quantified with a hematocytometer.

Western blot analysis

Cells were incubated in RIPA lysis buffer on ice for 30 min to lyse the cells. After centrifugation, the protein concentration in the supernatant was determined using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA). Protein lysates were separated on SDS-PAGE gels (10%) and transferred onto polyvinylidene difluoride membranes (PVDF). Membranes were probed overnight with the following antibodies: ERα (1:1000), Bcl-2 (1:500), Bax (1:1000), and GAPDH (1:5000). The membranes were incubated with the respective secondary antibodies for 1 h, and antigens were detected by enhanced chemiluminescence.

Statistical analysis

All statistical analyses were done using SPSS for Windows version 15.0. Statistical differences between multiple groups were tested using analysis of variance (ANOVA). Post hoc testing was performed with the Bonferroni method. All experiments were performed independently for at least three times and in triplicate for each time. Results were presented as mean ± standard error of the mean (SEM).A p value of 0.05 was considered significant.

Abbreviations

ERα: estrogen receptor alpha; E2: 17-beta estradiol; MTT: 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; PI: Propidium iodide; BC-ER cells: ERα transfected Bcap37 cells; BC-V cells: Bcap37 cells transfected with empty vector.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

LW and MJ contributed to the conception and design of the study, data interpretation. ZJ and JG performed experiments, analyzed data, and drafted the manuscript. SX performed experiments and analyzed data. JS helped to design statistical approaches and analyzed data. All authors read and approved the final manuscript.

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